Ann's Lab Notebook

Aug 5

Prepare samples for sequening. Are our constructed parts wrong because of us or iGEM kit? If us, where do we restart?

Task 1: Check Kit

To make sure we took out the samples we intended to.

	1	2D	K325219	Red Luciferase	Ours
	1	2D	K325219	Red Luciferase	Fudan
	1	2F	K325108	EPIC Luciferase	Ours
	1	2F	K325108	EPIC Luciferase	Fudan
	1	2H	K325209	Green Luciferase	Ours
	1	2H	K325209	Green Luciferase	Fudan
	1	2J	K325259	Yellow Luciferase	Ours
	1	2J	K325259	Yellow Luciferase	Fudan
	1	4J	K325218	Orange Luciferase	Ours
	1	4J	K325218	Orange Luciferase	Fudan
	1	19E	K592009	amilCP blue chromprotein	
	2	3F	K880005	p/rbs1	
	3	6C	B0017	double terminator	
	3	16E	K081005	constitutive p + RBS 1	
	4	2I	K592011	cjBlue green chromoprotein	
	4	6K	K1033910	fwYellow chromoprotein	
	4	6M	K1033916	amajLime chromoprotein	
	4	1B	B0011	strong terminator 1	
	4	1F	B0025	double terminator (reversed)	
	4	1D	B0024	double terminator (reversed)	
	4	16G	B0010	T1 from E. coli rrnB	
	5	3J	K1486022	Renilla Luciferase	
	5	5F	K1467201	ggBLue chromoportein	

Task 2: Prep miniprepped plasmids

Requirements:

1. Dissolve purified DNA in 30~50 ul ddH2O
2. Concentration of plasmid should be >100-200 ng/ul

	K325259	Yellow Luciferase	posT	95.4
	K325259	Yellow Luciferase	fudan	68.2
	K1033910	fwYellow chromoprotein	posT?	137.3
	B0017	double terminator	posT	107
	K081005	constitutive p + RBS 1	posT?	345
	K1467201	ggBLue chromoportein	Fudan?	157.4
	B0011	strong terminator 1	Fudan?	82.5
	minipreppe	p/rbs + lime 1		153
	minipreppe	p/rbs + ggblue		114.3

Task 3: Check plated colonies

Task 4: Transform rehydrated DNA

All parts to be sequenced

	1	2D	K325219	Red Luciferase	Plate
	5	3J	K1486022	Renilla Luciferase	Plate
	1	2F	K325108	EPIC Luciferase	Plate
	1	2F	K325108	EPIC Luciferase	Fudan
	1	2H	K325209	Green Luciferase	Plate
	1	2H	K325209	Green Luciferase	Fudan
	1	4J	K325218	Orange luciferase	Plate
	1	4J	K325218	Orange luciferase	Fudan
	1	2J	K325259	Yellow Luciferase	plate preT
	1	2J	K325259	Yellow Luciferase	plate PosT
	1	2J	K325259	Yellow Luciferase	Fudan
	1	19E	K592009	amilCP blue	PreT
	4	2I	K592011	green chromoprotein	PreT
	4	6K	K1033910	fwYellow chromoprotein	RS
	4	6K	K1033910	fwYellow chromoprotein	Plate
	4	6K	K1033910	fwYellow chromoprotein	Fudan
	4	6M	K1033916	amajLime chromoprotein	RS
	4	6M	K1033916	amajLime chromoprotein	Plate
	4	6M	K1033916	amajLime chromoprotein	Fudan
	5	5F	K1467201	ggBLue chromoportein	RS
	5	5F	K1467201	ggBLue chromoportein	Plate
	5	5F	K1467201	ggBLue chromoportein	Fudan
	3	6C	B0017	double terminator	PosT
	3	16E	K081005	constitutive p + RBS 1	PosT
				p/rbs + lime	
				p/rbs + ggblue	

	1	2D	K325219	Red Luciferase	Fudan
	1	2D	K325219	Red Luciferase	PosT
	1	2F	K325108	EPIC Luciferase	PosT
	1	2H	K325209	Green Luciferase	PreT
	1	2H	K325209	Green Luciferase	Fudan
	1	2J	K325259	Yellow Luciferase	PreT
	1	4J	K325218	Orange luciferase	Fudan
	1	19E	K592009	amilCP blue chromprotein	PreT
	2	3F	K880005	p/rbs1	PreT
	3	6C	B0017	double terminator	PosT
	3	16E	K081005	constitutive p + RBS 1	preT
	4	2I	K592011	cjBlue green chromoprotein	Fudan
	4	2I	K592011	cjBlue green chromoprotein	PreT
	4	6K	K1033910	fwYellow chromoprotein	PreT
	4	6M	K1033916	amajLime chromoprotein	PreT
	4	6M	K1033916	amajLime chromoprotein	Fudan
	4	1B	B0011	strong terminator 1	PreT
	4	1F	B0025	double terminator (reversed)	PreT
	4	1D	B0024	double terminator (reversed)	PreT
	4	16G	B0010	T1 from E. coli rrnB	PreT
	5	3J	K1486022	Renilla Luciferase	PreT
	5	5F	K1467201	ggBLue chromoportein	PreT

Aug 6

Prepare primers

Primer	Stock Solution	PCR (10μM)
Blue Forward	110μL for 100μM	Dilute 10μL to 100μL
Blue Reverse	158μL for 100μM	Dilute 10μL to 100μL
Lime Forward	92μL for 100μM	Dilute 10μL to 100μL
Lime Reverse	146μL for 100μM	Dilute 10μL to 100μL
Yellow Forward	92μL for 100μM	Dilute 10μL to 100μL
Yellow Reverse	167 for 100μM	Dilute 10μL to 100μL

Nanodrop Primers

Results came out funny: 1000ng/uL or 3000ng/uL. We would expect a concentration of 100uM (where 33ug/ml = 0.1mM). Thus 100uM = 0.1mM = 33ng/uL.

Prepare IDT DNA

1000ng —> 100ng/uL
Using 3uL: 100ng/uL —> 10ng/uL

PCR

Reaction Protocol

Mix on ice and quickly transfer to thermocycler heated to 98

Component	50μL Reaction	Concentration
5X Q5 Reaction Mix	25μL	1X
10uM Forward primer	2.5μL	0.5μM
10uM Reverse primer	2.5μL	0.5μM
Template DNA (1)	10μL	100ng
Nuclease-free water	10μL or none	

Notes: I realized I put 10ng/uL primer, not 10uM. 10uM should be 3.3ng/uL concentration…

Reaction Conditions

Initial Denaturation	98°C	30 seconds
25 cycles	98°C	10 seconds
	59°C (blue)	15 seconds
	59°C (lime)	15 seconds
	58°C (yellow)	15 seconds
	72°C	40 seconds
Final Extension	72°C	2 minutes
Hold	4-10°C	

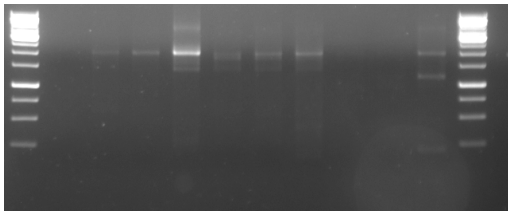
1 Blue 55.7 (E)	1 Blue 59.1 (D)	1 Blue 62 (C)
Yellow 55.7 (E)	Yellow 59.1 (D)	1 Yellow 62 (C)
Lime 55.7 (E)	Lime 591 (D)	2 Lime 62 (C)

Check transformation plates

All growth. Inoculated at 4pm. 12mL SOC, 12mL chloramphenicol, 1 colony bacteria.

Run Gel to check PCR

Ladder	(-)	Yell 62	Yell 59	Yell 56	Lim 62	Lim 59	Lim 56	Blu 62	Blu 59	Blu 56	Ladder



Miniprep inoculated cultures

Didn't get to nanodrop.

Aug 7

Nanodrop Yesterday's Miniprep

Miniprep inoculated samples

Nanodrop miniprepped-samples results

PCR purification

Prep

- add ethanol to buffer PW (see bottle for volume)
- Calculate volume of buffer EB
- Dissolve precipitates in buffer BL in 37C for several minutes

Equilibriate

Add 500 uL Buffer BL to spin column CB2
Centrifuge 1min at 12,000rpm. Discard flow through.

Bind

Add 5 volumes of Buffer PB to 1 volume of PCR reaction. Mix.
225uL Buffer PB : 45uL PCR
Apply mixture to column
Let stand for 2min
Centrifuge 45s at 12,000rpm. Discard flow through

Wash

Add 600uL Buffer PW to spin column
Let stand 2-5min
Centrifuge 45s at 12,000rpm. Discard Flowthrough
Repeat
Centrifuge at 12,000rpm for 2min to remove residual Buffer PW
Discard flow and allow column to dry with cap open for 3min

Elute

Place spin column in clean microcentrifuge tube. Add 30-50uL Buffer EB to center.
Incubate 2 min.
Centrifuge 12,000rpm for 2min.

Nanodrop purification results

Y62: 28.8ng/uL
260:280: 2.77

Re-PCR: for less nonspecific bands

Component	50μL Reaction	Concentration
5X Q5 Reaction Mix	25μL	1X
10uM (3.3ng/uL) F primer	2.5μL	0.5μM
10uM (3.3ng/uL) R primer	2.5μL	0.5μM
Template DNA	1μL	10ng
Nuclease-free water	19μL	

Notes: For low complexity templates (i.e. plasmid), use 1 pg–10 ng of DNA per 50 μl reaction

Prepare primer: Currently 92.5uL of 10ng/uL concentration.
Take 5uL out. Add 10uL. Dilute to 3.3ng/uL.

Prepare template DNA. 1uL of 100ng/uL DNA. Dilute to 10ng/uL by adding 9uL water.

Blue 59.5(D)	Blue 56.3 (E)	Blue 53.7 (F)
Yellow 59.5 (D)	Yellow 56.3 (E)	Control 53.7 (F)
Lime 59.5 (D)	Lime 56.3 (E)	

Initial Denaturation	98°C	30 seconds
25 cycles	98°C	15 seconds
	59°C (blue)	30 seconds
	59°C (lime)	30 seconds
	58°C (yellow)	30 seconds
	72°C	60 seconds
Final Extension	72°C	2 minutes
Hold	4-10°C	

Conclusion: Barely cleaner. Seems like bands slightly brighter. Bands used for extraction were 56C annealing temperature.

Restriction Digest

1uL EcoRI
1uL PstI
5uL NEBBuffer 3.1
1ug DNA
(For Y56: 57ng/uL —> 0.5 digest dilution —> 25uL)
(For L56: 44.2ng/uL —> 0.5 digest dilution —> 25uL)
(For B56: 42ng/uL —> 0.5 digest dilution —> 25uL)
50uL total reaction volume
1hr incubation time at 37°C.
Heat inactivation 80C 20min

Aug 10

Prep

Prepare 42C, 65C water bath
Check SOC amounts
Check backbone from inventory + thaw
Check agar plates for that antibiotic resistance
Thaw 250uL competent cells on ice
Prepare 0.75% gel

Restriction Digest for more backbone

2PCR tubes labeled C. backbone 1 + 2
1uL EcoRI
1uL PstI
5uL NEBBuffer 3.1
1ug DNA
50uL total reaction volume
Thermal cycler. 1hr incubation time at 37°C. Heat inactivation 80C 20min

Ligation of PCR purified products (room temp)

5 microtubes labeled Y56, L56, B56, C+L, C-L
Use backbone from inventory

10X T4 DNA Ligase Buffer	2uL
Vector DNA (37.5ng)	2.08uL / 2.88 for lime
Insert DNA	3:1 insert to vector ratio
Yellow56	3.81uL
Lime56	4.92uL
Blue56	5.18uL
Control	Cut vector + ligase (background due to vector recircularization)
Control	Cut vector - ligase (background due to uncut vector)
ddH2O	to 20uL
T4 DNA Ligase	1uL

Incubate at room temperature for 10min
Heat inactivate at 65°C for 10 min.
Chill on ice and transform 1-5 µl into 50µl competent cells.

Gel electrophoresis of PCR results

Run for extraction. 0.75%. Run until blue marker is 2/3 down. (45min?)

Gel Extraction

Notes

If a large amount of DNA is purified or if the volume of the binding reaction is greater than 1.5 ml increase the incubation time of the binding step to 15 min.

Prep

Prepare 60C water bath
Heat elution buffer to 60 before use

Equilibriate

Add 500uL Buffer BL to spin column CA2.
Centrifuge 1min at 12,000rpm. Discard flow through.

Excise

Excise gel slice.
Weigh the gel slice in a preweighed 1.5ml tube.

Dissolve
Add 3:1 volume of Buffer PN to gel (300uL Buffer / 100mg gel)
Incubate at 60C for 5min (gel slice is dissolved)
Mix inversion every few min to facilitate melting.
Check color. Yellow is optimal pH. If orange or violet, add 10ul of 3M sodium acetate, pH 5.2 and mix
Cool solution to room temperature 5min

Bind
Pour solution to CA2 column and let stand for 2min.
Centrifuge 45seconds at 12000 rpm. Discard flow through. For critical samples, repeat centrifuge.

Wash
Add 600ul Buffer PW and wait for 2~5 minutes
Centrifuge 1min at 12000 rpm. Discard flow through.
Repeat
Centrifuge at 13000 rpm for 10 min to spin the ethanol down.
Put the column into a fresh tube. If necessary air-dry the pellet for 10-15 min to avoid the presence residual ethanol

Elution
Add 30-50ul elution buffer (EB)
Place in room temperature for 2min.
Centrifuge 12000 rpm for 2min
Add the DNA solution back to column and centrifuge again.

Nanodrop
DNA should have a peak at OD260.

Restriction Digest of Gel Products

0.2uL EcoRI
0.2uL PstI
2uL NEBBuffer 3.1
200ug DNA
20uL total reaction volume
1hr incubation time at 37°C.
Heat inactivation 80C 20min

Ligation of PCR purified products (overnight)

10X T4 DNA Ligase Buffer	2uL
Vector DNA (37.5ng)	1.637uL
Insert DNA	3:1 insert to vector ratio
Yellow56	3.81uL
Lime56	4.92uL
Blue56	5.18uL
Control	Cut vector + ligase (background due to vector recircularization)
Control	Cut vector - ligase (background due to uncut vector)
ddH2O	to 20uL
T4 DNA Ligase	1uL

Incubate at 16°C overnight.
Heat inactivate at 65°C for 10 minutes.
Chill on ice and transform 1-5 µl into 50µl competent cells.

Transformation of first ligation

Add 2 μL of the resuspended DNA to the competent cells.
Incubate on ice for 30 minutes.
Heat shock at 42ºC for 60 seconds.
Incubate the cells on ice for 5min.
Add 200 µl of SOC media
Incubate cells at 37ºC for 2 hours
Plate 225 μl of the transformation onto the labeled antibiotic dishes
Spread remaining transformation onto LB agar plate
Incubate the plates at 37ºC overnight

Aug 11

Check Transformation Products!

Do you see color? please please please…

No color. Tried inoculating a colony into 4mL SOC

SOC CONTAINS GLUCOSE WHICH INHIBITS pBAD PROMOTER>>>SHIT

Take Ligation out and put in freezer

Check backbone integrity

Didn't need. Vector+ligase control results came out good (only a few colonies)

Design primers for amplifying linear backbone

Not yet.

Transformation

Of Ligation products. (YLB PCR'd ligated overnight DNA)
Unfortunately done in SOC media, so no color expected. Plated concentrated arabinose solution on top of plate first, but left a very liquidy solution. No colonies expected.

Inoculation of transformation products into LB medium

Y1 used 10mL LB, 10uL chloramphenicol, 100uL of 500mg/mL arabinose

Y2 used 10mL LB, 10uL chloramphenicol, 100uL of 2mg/mL arabinose

B56 used 10mL LB, 10uL chloramphenicol, 100uL of 500mg/mL arabinose

Prepare samples for sequencing

1. Spencer's construct
2. 3 IDT constructs
3.

Aug 12

Objective

- To miniprep samples to send for sequencing
- To play around with arabinose concentrations in order to find the best for chromoprotein expression.
- To redo transformation with LB media

When you enter the lab.

Turn on UV.
Prepare 42C water bath.

Miniprep inoculated samples to send to IDT

In the shaker, there are 3 LB-based yellow 1, yellow 2, blue. Miniprep these. There is 10mL of LB in there, where as the other tubes will only have about 4mL of SOC.

Fill out form for sending the samples

We should be sending a total of four samples: (1) Yellow 1 (2) Yellow 2 (3) Blue (4) Spencer's construct.

Primers

VF2 - 5' TGCCACCTGACGTCTAAGAA 3'
VR - 5' ATTACCGCCTTTGAGTGAGC 3'

Type:
测通
NOT 双向or单向

Plasmid 质粒
pSB1C3

Organism
E.Coli

Sequence Size
~2kb for IDT sequences
~1kb for spencer's construct

Names:
A1, A2, A3, A4? Idk pick whatever is easy to remember and save it somewhere.

Make LB Broth. Make a good jar full.

Total Amoung of Reagent:	100mL	250 mL
Deionized Water	100mL	250mL
Yeast	1g	2.5g
Tryptone	0.5g	1.25g
NaCl	1g	2.5g

SOC Medium

Try another round of inoculation from the plates in the incubator USE LB.

Not overnight inoculation, but ~6hrs.

Each inoculation tube should have:
10mL LB
10uL Chloramphenicol
1 colony bacteria
Make 4 liquid cultures for each plate.

Yellow 1 0mM LB 8/12	Yellow 1 1mM LB 8/12	Yellow 1 10mM LB 8/12	Yellow 1 33mM LB 8/12
Yellow 2 0mM LB 8/12	Yellow 2 1mM LB 8/12	Yellow 2 10mM LB 8/12	Yellow 2 33mM LB 8/12
Blue 0mM LB 8/12	Blue 1mM LB 8/12	Blue 10mM LB 8/12	Blue 33mM LB 8/12

Put these in the shaker at 230rpm. In 2hrs time, check the OD of one of the samples. If OD600 ~ 0.5, then add arabinose. Check these add the end of the day. If still no color, continue shaking overnight.

Make stock solution of arabinose.
Concentration: 200mg/mL
20mL water
4g arabinose
Using info from here: http://biodisplay.tyrell.hu/implementation/protocols/basic-protocols/

For arabinose concentrations use these: assuming stock concentration 200mg/mL

	0mg/mL arabinose	1mM = 1.5mg/ 10mL	10mM = 15mg/ 10mL	33mM = 50mg/ 10mL
Yellow (1)	0	7.5uL	75uL	300uL
Yellow (2)	0	7.5uL	75uL	300uL
Blue	0	7.5uL	75uL	300uL

Example calculation of mM
5mM = (5e-3 mol/L)
10mL = 0.01L
molar mass of arabinose = 150g/mol = 150000mg/mol

(5e-3 mol/L)(0.01L)(150,000mg/mol) = 7.5mg

Another transformation
Using LB media. Not SOC.

In the small PCR tubes labeled 1 2 3 4 5, those are all ligation products.
1: Yellow
2: Lime
3: Blue

For the other ligation products, the date would be Aug. 10. They would be labeled yellow + bb or something of that nature. They might be in the ligation box. Or they are in a blue tube holder (long rectangle) close to the original IDT samples. There would be another three transformations with these ligation products (yellow, lime blue)

Thus a total of 6 transformations.

Play around with adding arabinose to the plate and letting it sit just before adding bacteria.

Aug 17

Remake Spencer's Part

Add a terminator. Digest, ligation, transformation. Use low copy plasmid

Making more backbone:

10uL backbone
(137.8ng/uL psB1A3)
(132.1ng/uL psb1C3)
1uL EcoRI
1uL PstI
5uL NEB buffer 3.1
33uL water

Digest Spencer's Part

25uL Part
(137.5ng/uL)
1uL EcoRI
1uL XbaI
5uL NEB buffer 3.1 37C
18uL water

Add 8uL for ligation

Digest Terminator Part

25uL terminator
(50.8ng/uL)
5uL Neb buffer 1.1 or 2.1 at 37C
1uL SpeI
1uL PstI
18uL water

GEL
C1 C2 A1 A2

Only used C2, A1, A2
[C1 260:280 was about 2.7, concentration below 10ng/uL]
See google drive for concentrations.